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Dynamic Covalent Transport of Amino Acids across Lipid Bilayers

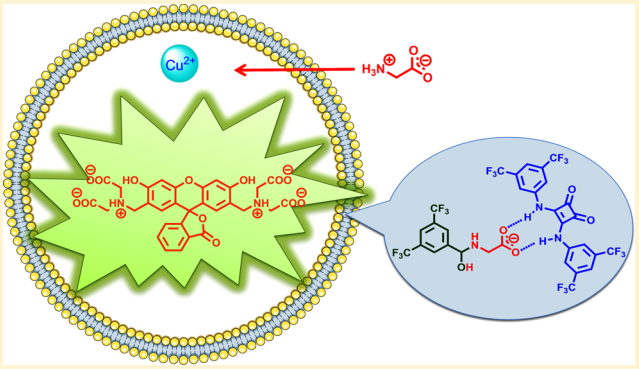
Xin Wu,[†] Nathalie Busschaert,[†] Neil J. Wells,[†] Yun-Bao Jiang,[‡] and Philip A. Gale*[,†]

[†]Chemistry, University of Southampton, Southampton, SO17 1BJ, U.K.

[‡]Department of Chemistry, College of Chemistry and Chemical Engineering, and the MOE Key Laboratory of Spectrochemical Analysis and Instrumentation, Xiamen University, Xiamen 361005, China

Supporting Information

ABSTRACT: We report a dynamic covalent approach to transmembrane transport of amino acids by the formation of a three-component assembly. A mixture of a squaramide and a lipophilic and electrophilic aldehyde is shown to synergistically transport highly polar glycine (Gly) across vesicle membranes. The transport was investigated by a ^{13}C NMR assay, an osmotic response assay, a newly developed fluorescence assay suitable for measuring Gly influx, and other fluorescence assays for leakage and pH change. The transport is proposed to occur via a hydrogen-bonded anionic glycine hemiaminal/imine, accompanied by transport of OH^- in the opposite direction. Several control experiments support the role of hemiaminal/imine in the observed facilitated Gly transport. Proton NMR studies of a biphasic system show the presence of both the hemiaminal and imine formed between Gly and an aldehyde. Interestingly, the synergistic effect has also been observed for sarcosine, which can form hemiaminals but not imines. The results demonstrate the potential of hemiaminal formation for the facilitated transport of substrates containing primary and secondary amino groups.

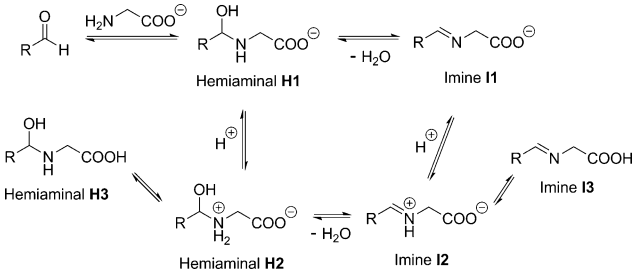


INTRODUCTION

There is currently considerable interest in developing synthetic systems[1] to mediate the transport of highly hydrophilic species, e.g., cations, anions, sugars, and amino acids across lipid bilayers, functioning as either a membrane-spanning channel[2] or a mobile carrier.[3] Such systems could perform the function of naturally occurring transmembrane proteins and could serve as important tools for biomedical research or have therapeutic potential.[4] For those functioning as mobile carriers, a dynamic interaction is responsible for reversibly binding and lipophilizing substrates, allowing the lipophilic complex to diffuse through the membrane and dissociate to release the transported substrate into the aqueous phase.[3] Noncovalent interactions including ion-pairing,[5] hydrogen bond,[6] halogen bond,[7] metal coordination,[8] and cation[9]/anion[10]−π interactions are well-exploited. Dynamic covalent bonds,[11] in contrast, have been rarely used as the interaction for reversible binding of the transported substrates. Smith and co-workers have reported synthetic transporters for saccharides[12] and ribonucleosides[13] based on boronate ester formation. Stillwell has studied facilitated diffusion of amino acid across planar lipid bilayers using water-soluble aldehydes that presumably form imines with amino acids,[14] but the activity was low even with ~10 mM aldehyde concentrations. Matile and co-workers reported cellular delivery of thiol-containing substrates based on in situ formation of cell-penetrating poly(disulfide)s (CPDs).[15] The CPD systems are, however, conceptually different from the other above-mentioned examples, as they require intracellular glutathione (GSH) to break the disulfide bonds and release the substrate.

Hemiaminals are labile adducts formed between carbonyl compounds and primary or secondary amines (Scheme 1).

Scheme 1. Formation of Hemiaminals and Imines between an Aldehyde and Glycine



Compared to imine chemistry, hemiaminals are less explored in the context of dynamic covalent chemistry due to their instability, while stabilization can be afforded by using highly electrophilic carbonyl compounds[16] or a further binding event.[17] The biological relevance of hemiaminals is not limited to the involvement in imine formation. It has been reported that hemiaminal formation is used by *Escherichia coli*

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dihydroxyacetone kinase for substrate binding.[18] We report here that hemiaminal formation with lipophilic and highly electrophilic aldehydes can be used to facilitate the transport of polar amino acids through lipid bilayer membranes.

Amino acid transport is crucial to the proper function of the nervous system,[19] absorption of nutrients,[20] and cell volume regulation.[21] There are currently only a few reported investigations of synthetic amino acid transporters that function in lipid bilayers,[22] although some systems are reported that transport amino acids through an organic liquid phase.[23] We demonstrate herein facilitated transport of glycine (Gly) across vesicle membranes by formation of a dynamic lipophilic three-component assembly using a squaramide[6c] intended for hydrogen bonding to the Gly carboxylate group and an aldehyde for imine/hemiaminal formation (Scheme 1) with the Gly amino group. A mixture of the squaramide **1** and a lipophilic aldehyde **2** or **3** (Figure 1) is found to facilitate Gly

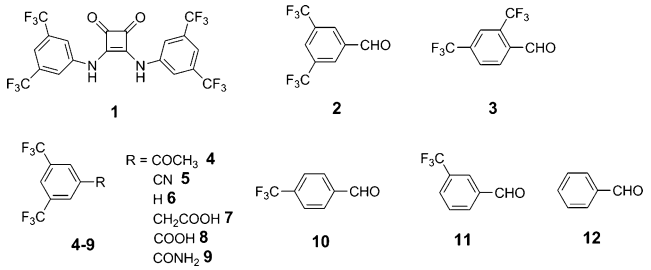

Figure 1. Structures of compounds tested for Gly transport.

transport with good activity, with the two components working in a synergistic manner, as evidenced by a ^{13}C NMR assay, an osmotic response assay, and a newly developed fluorescence assay in vesicle-based experiments. The inability of several control compounds to transport Gly and of the aldehydes to transport *N*,*N*-dimethylglycine, oxalic acid/oxalate, and calcein provides strong evidence of Gly transport by aldehyde carbonyl addition, while similar synergistic transport observed for sarcosine (Sar) indicates that reversible hemiaminal formation is sufficient for facilitated transport without the need to form imines, a hypothesis that is also supported by ^{1}H NMR studies of a biphasic system. The transport mechanism is proposed as a $H_2NCH_2COO^-/OH^-$ antiport.

RESULTS AND DISCUSSION

^{13}C NMR Assay. Evidence for glycine transport facilitated by a mixture of an aldehyde and a squaramide has been afforded by a ^{13}C NMR assay[24] (Figure 2). Gly-1-^{13}C was added externally to a suspension of giant multilamellar vesicles (GMLVs) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and DMSO solutions of an aldehyde and/or a squaramide (or DMSO as control) were added to start the Gly influx. After 2 h, paramagnetic Mn^{2+} was added to quench the ^{13}C NMR signal from extravesicular Gly, and then the ^{13}C NMR spectra of the vesicle suspension were measured. The results show that without any transporter, there is only very slow, simple diffusion of Gly. An enhanced signal from the intact Gly-1-^{13}C was observed with only **1** (1 mol % transporter to lipid) or **2** (10 mol %) alone, but a mixture of **1** and **2** induced ca. 50% more glycine transport than the sum of that induced by each component, as estimated by integration of the intravesicular Gly signal (Figure S2, Supporting Information). A similar synergistic effect in facilitating Gly transport by **1** and **3**

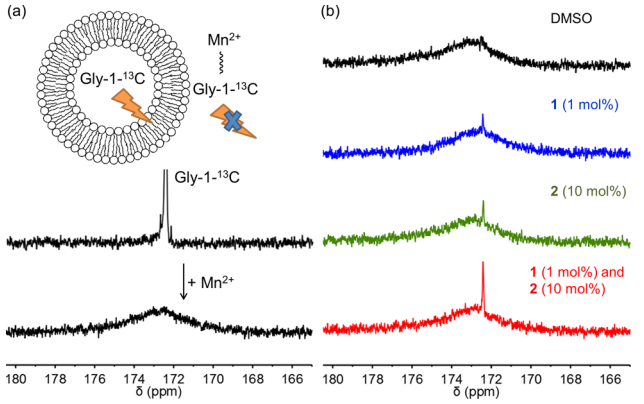

Figure 2. (a) Schematic representation of the ^{13}C NMR assay for Gly transport. POPC GMLVs were loaded with an internal solution of Na_2SO_4 (100 mM) and HEPES (20 mM) buffered at pH 7.4 and suspended in an external solution of Gly-1-^{13}C (50 mM), Na_2SO_4 (100 mM), and HEPES (20 mM) buffered at pH 7.4 in 9:1 (v:v) H_2O-D_2O. $MnSO_4$ was added to the external medium (0.5 mM), which led to broadening of ^{13}C NMR signal from Gly-1-^{13}C. (b) ^{13}C NMR spectra (100.6 MHz) of POPC vesicles solutions in 9:1 (v:v) H_2O-D_2O with external Gly-1-^{13}C (50 mM) before and after addition of $MnSO_4$ (0.5 mM). (c) ^{13}C NMR spectra of POPC vesicles suspensions with 50 mM external Gly-1-^{13}C, obtained 2 h after the addition of DMSO or DMSO solutions of transporters. Transporter loadings are shown as transporter to lipid molar ratios.

was also observed (Figures S1 and S2, Supporting Information).

Fluorescence Assay. Due to the long acquisition time required, NMR assays are not suitable for quantitative kinetic studies. Previously amino acid transport kinetics has been measured using a radiometric assay[25] and a fluorescence labeling method.[26] However, both assays are expensive and time-consuming and only allow collection of a limited number of data points, which can be problematic when measuring very rapid transport. Here we develop and employ a novel fluorescence assay for amino acid influx using vesicles with an entrapped Cu^{2+}–calcein complex. This is based on fluorescence quenching of calcein by binding to Cu^{2+} [27] and the recovery of calcein fluorescence by amino acids that rapidly compete with calcein for Cu^{2+}.[28] The membrane impermeability, long-wavelength excitation, and high fluorescence quantum yield of the calcein dye are favorable for sensing of amino acids inside vesicles. Large unilamellar vesicles (LUVs, mean diameter 200 nm) of POPC entrapping calcein (0.2 mM) and Cu^{2+} (0.2 mM) were prepared and separated from the extravesicular markers by gel filtration. The vesicles were suspended in an external solution containing Gly (30 mM) and Cu^{2+} (0.2 mM) buffered at pH 7.4, and then DMSO solutions of transporters or DMSO were immediately added to the vesicle suspensions to start the transport. It was found that the presence of external Cu^{2+} is necessary to suppress Cu^{2+} efflux from inside vesicles, which would otherwise occur in an overwhelming manner even without any transporter when high concentrations of external Gly is present (Figure S5, Supporting Information). Even with external Cu^{2+}, when the simple diffusion or Gly-mediated diffusion of Cu^{2+} is suppressed, the addition of a transporter could still facilitate Cu^{2+} efflux (due to the difference between internal and external free Cu^{2+} concentrations when external Gly is present), leading to fluorescence enhancement that does not arise from Gly influx. Cu^{2+} transport, however, can be identified easily by doubling the concentration of extravesicular

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Cu^{2+}, and significant dependence of calcein fluorescence on external Cu^{2+} concentration is an indication of Cu^{2+} transport. This effect is clearly observed (Figure 3a) with 8-hydroxyquinoline (**8HQ**), a known Cu^{2+} transporter.[29] In contrast, a mixture of **1** and **2** or **3** induced enhancement of calcein fluorescence (ΔI) that is practically independent of the external Cu^{2+} concentration [Figures 3b and S21c (Supporting Information)], indicating that Gly influx is responsible for the observed fluorescence enhancement, which is also supported by the results of the ^{13}C NMR assay (Figure 2). Similarly, without any transporter, a relatively small enhancement of calcein fluorescence independent of external Cu^{2+} concentration is observed [Figures 3 and S5 (Supporting Information)] and is attributed to simple diffusion of Gly,[26,30] which is well-documented. The combination of 1 mol % (transporter to lipid) of **1** and 50 mol % of **3** leads to saturation of the fluorescence intensity within 20 min, and thus, the maximum fluorescence intensity enhancement obtained under this condition (ΔI_{max}) is used for calibration. The use of a high loading of **3** here is to ensure that completion of Gly transport is reached and clearly shown, while the facilitated transport is observable at much lower loadings. Since the ΔI_{max} value shows a roughly linear relationship with the extravesicular Gly concentration (Figure S6, Supporting Information), the $\Delta I/\Delta I_{max}$ value, here defined as the fractional fluorescence intensity (I_f), can be approximated as the percentage of Gly transported relative to the maximum transport.

Similarly to the results obtained with the ^{13}C NMR assay, the Cu^{2+}–calcein assay demonstrates a synergistic effect between the squaramide **1** and the aldehydes **2** and **3** (Figure 4 and Table 1). This is evidence in support of glycine transport by hemiaminal/imine formation with the amino group and hydrogen bonding with the carboxylate group. Presumably, the observed synergistic effect results from the higher lipophilicity of a ternary squaramide–Gly–aldehyde complex compared to binary squaramide–Gly and aldehyde–Gly conjugates. Note that the relative rate of simple Gly diffusion measured in the ^{13}C NMR assay with GMLVs is slower than that measured in the Cu^{2+}–calcein assay with LUVs, which is consistent with the lower permeability of MLVs.[31]

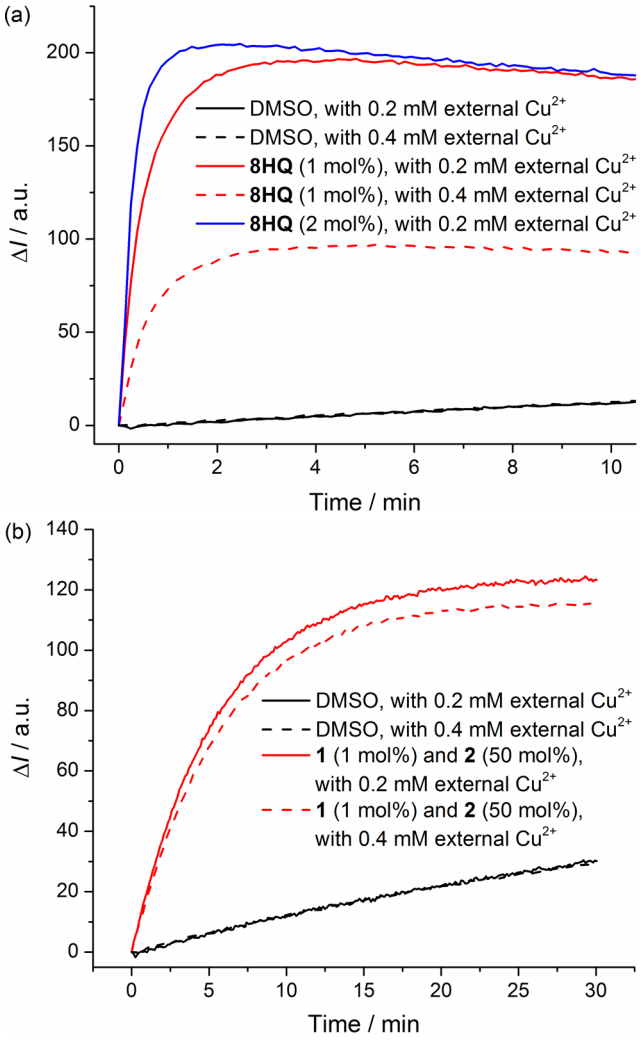

Figure 3. Evidence of Cu^{2+} transport in the case of **8HQ** (a) and Gly transport in the case of a mixture of **1** and **2** (b). POPC LUVs (mean diameter 200 nm) loaded with $CuSO_4$ (0.2 mM) and calcein (0.2 mM) were suspended in an external solution containing Gly (30 mM) and $CuSO_4$ (0.2 or 0.4 mM). Both the internal and external solutions contained Na_2SO_4 (100 mM) and HEPES (20 mM) buffered at pH 7.4. At time 0, DMSO solutions of transporters or DMSO was added, and the fluorescence intensity (λ_{ex} = 495 nm, λ_{em} = 515 nm) was recorded. Transporter loadings are shown as transporter to lipid molar ratios. Note that in part a, doubling the **8HQ** loading (from 10 to 20 μM) did not significantly alter the maximum ΔI value, indicating that the fluorescence enhancement by **8HQ** is not due to competitive binding of **8HQ** to Cu^{2+}. The much higher saturated ΔI value in part a compared to that in part b indicates that Cu^{2+} efflux occurs in the presence of **8HQ**. Although it is possible that the Cu^{2+}–**8HQ** complex also transports Gly, the results in part a show that the Cu^{2+}–calcein assay is not suitable when a Cu^{2+} transporter is present.

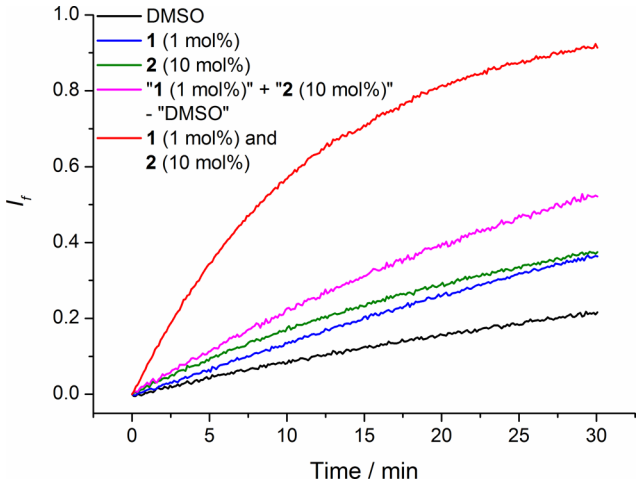

Figure 4. Gly transport kinetics measured by the Cu^{2+}–calcein assay. POPC LUVs (mean diameter 200 nm) loaded with $CuSO_4$ (0.2 mM) and calcein (0.2 mM) were suspended in an external solution containing Gly (30 mM) and $CuSO_4$ (0.2 mM). Both the internal and external solutions contained Na_2SO_4 (100 mM) and HEPES (20 mM) buffered at pH 7.4. At time 0, DMSO solutions of transporters or DMSO was added, and the fluorescence intensity (λ_{ex} = 495 nm, λ_{em} = 515 nm) was recorded. The fluorescence intensity was normalized by saturation using a mixture of **1** (1 mol %) and **3** (50 mol %). Transporter loadings are shown as transporter to lipid molar ratios.

Osmotic Response Assay. It is possible that Cu^{2+} enhances Gly transport by binding to Gly, the hemiaminal, the imine, or the squaramide **1**, even though the kinetic profiles with different Cu^{2+} concentrations (Figure 3b) is evidence that

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Table 1. Gly Transport Properties and Calculated log *P* (cLogP) Values of 1−11

	without **2**		with **2** (10 mol %)		
	EC_{50}[a]/mol %	*n*	EC_{50}[a]/mol %	*n*	cLogP[d]
1	~8[b]	~0.85[b]	0.1	0.96	6.94
	without **1**		with **1** (1 mol %)		
	EC_{50}[a]/mol %	*n*	EC_{50}[a]/mol %	*n*	cLogP[d]
2	56	0.94	1.9	1.1	3.58
3	63	0.92	4.2	1.5	3.86
4	>100	nd[c]	>100	nd[c]	4.06
5	>100	nd[c]	>100	nd[c]	3.74
6	>100	nd[c]	>100	nd[c]	3.64
7	>100	nd[c]	>100	nd[c]	2.93
8	>100	nd[c]	>100	nd[c]	4.28
9	>100	nd[c]	>100	nd[c]	3.13
10	47	0.88	23	1.1	2.61
11	148	0.93	45	0.89	2.47
12	>100	nd[c]	>100	nd[c]	1.64

[a]Transporter loading (transporter to lipid molar ratio) needed to observe 50% transport at 30 min. [b]Small extent of Cu^{2+} transport found by **1** (Figure S21a, Supporting Information), and therefore, the values should be regarded as approximate. [c]Not determined due to low activity. [d]cLogP values were calculated by ACD/Laboratories.

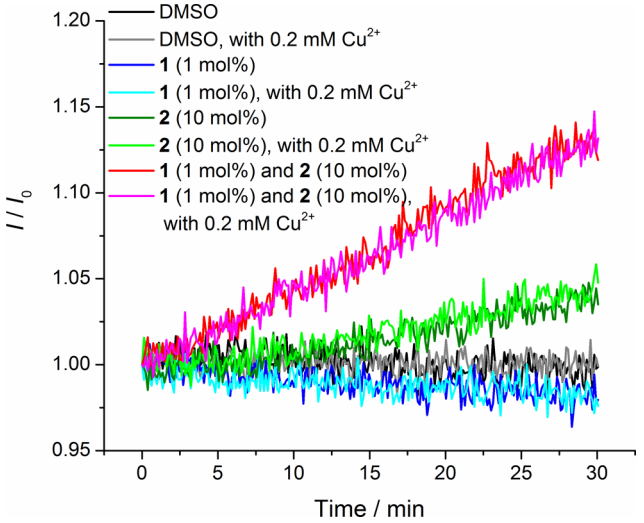

Figure 5. Gly transport measured by an osmotic response assay in the absence and presence of $CuSO_4$ (0.2 mM). POPC LUVs (mean diameter 400 nm) loaded with Gly (600 mM) were suspended in an external solution containing Na_2SO_4 (195 mM) and Gly (15 mM). Both the internal and external solutions were buffered at pH 7.4 with HEPES (20 mM). At time 0, DMSO solutions of transporters or DMSO was added, and the 90° light-scattering intensity at 600 nm was recorded. Transporter loadings are shown as transporter to lipid molar ratios.

leads us to suggest that this is unlikely. To examine the effect of Cu^{2+} on Gly transport, an independent osmotic response assay[32] was conducted. POPC LUVs (mean diameter 400 nm) loaded with 600 mM Gly were suspended in an isoosmotic external solution containing 195 mM Na_2SO_4 and 15 mM Gly. When Gly efflux occurs, the vesicles will become hyperosmotic, leading to water efflux and thus vesicle shrinkage, which results in an increase in 90° light-scattering intensity. Thus, Gly transport can be indirectly measured by monitoring the light-scattering intensity using a fluorimeter (Figure 5). Under the described experimental conditions, Gly transport by simple diffusion and facilitated by **1** (1 mol %) is too slow to generate an osmotic response. A delay is observed in the osmotic response induced by **2** (10 mol %), whereas the **1**−**2** mixture generates the osmotic response immediately after being loaded to the vesicle suspensions, showing the synergistic effect in Gly transport. Similar results have been obtained for **1** and **3** (Figure S3, Supporting Information). Importantly, the presence of Cu^{2+} has no observable effect on the rate of Gly transport (Figure 5), which confirms the validity of the Cu^{2+}−calcein assay in studying the squaramide−aldehyde system. No change in light-scattering intensity was found with vesicles with the same internal and external content, excluding aggregation or precipitation of the transporters as the cause for the observed increase in light-scattering intensity (Figure S4, Supporting Information). The osmotic response assay appears, however, not sufficiently sensitive for accurate kinetic studies.

Gly Transport Studies. Using the new Cu^{2+}−calcein assay, possible Gly transport by the nonaldehyde control compounds **4**−**9** were tested to provide more evidence for Gly transport by aldehyde carbonyl addition forming either hemiaminals or imines. First, the ketone analogue **4** was tested to investigate the possible role of the carbonyl group of an aldehyde as a hydrogen-bond acceptor for the ammonium or amino group of Gly. Little activity was found with **4** (Figure S8a, Supporting Information), ruling out this effect to be responsible for Gly transport. Second, **5**, with an electron-withdrawing cyano group, was tested for possible anion−π interaction in the case of **2** with the Gly carboxylate group. Compound **5** shows a far weaker transport activity (Figure S8b, Supporting Information), and therefore, the observed glycine transport with aldehyde cannot be due to an anion−π interaction. Third, the synergistic transport of Gly by the lipophilic aldehydes with **1** could arise from enhanced partition of **1** in the lipid in the presence of **2** or **3**. The results with compound **6** show this effect to be minor (Figure S8c, Supporting Information). Fourth, the aldehyde−Gly imine/hemiaminal formed in the lipid bilayer could serve as a Gly transporter by using its carboxylate group to ion pair with the ammonium group of the Gly substrate (inset in Figure S8d, Supporting Information). Compound **7**, which also contains an aliphatic carboxylate group, shows no facilitated Gly transport (Figure S8d, Supporting Information), thus ruling out that possibility. The facilitated Gly transport could also be due to aerobic oxidation of **2** forming a trace amount of aromatic carboxylic acid **8** that ion pairs with Gly, which is precluded as **8** fails to transport Gly (Figure S8e, Supporting Information). A sixth, less likely possibility is the aldehyde proton serving as a hydrogen-bond donor, which was also ruled out as the amide **9** shows no Gly transport activity (Figure S8f, Supporting Information).

It is possible that **1** facilitates Gly transport without binding to the carboxylate/carboxylic acid group and, instead, only binds and transports OH^- while the anionic hemiaminal/imine with a carboxylate group crosses the membrane without binding to **1**. In that case, **1** would simply serve as a protonophore that canceled the charge separation and pH imbalance due to the movement of anionic and basic NH_2COO^-. Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP),[33] which is an active protonophore but not a good hydrogen-bond donor, was tested as a negative control. CCCP itself induced negligible Gly flux, and it was unable to enhance Gly transport in the presence of **2** or **3** (Figure S9, Supporting Information), indicating that **1** needs to bind to the

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carboxylate/carboxylic acid group of Gly to facilitate Gly transport.

Less fluorinated aldehydes **10**–**12**, on the other hand, show appreciable transport activity. The aldehydes were tested at different loadings in the presence and absence of **1**, and the fractional fluorescence intensity at 30 min was plotted against the transporter loading and subjected to Hill analysis[34] to obtain the effective transporter loading needed to observe 50% transport at 30 min (EC_{50}) and a Hill coefficient (n) [Figures S10–S19 (Supporting Information) and Table 1]. The n values close to 1 are consistent with the 1:1 stoichiometry for the hemiaminal or imine formed between glycine and an aldehyde, evidence in support of a mobile carrier mechanism. Similarity, the n value for **1** in the presence of **2** (10 mol %) is close to 1. The n values are consistent with the stoichiometry of the assumed three-component aldehyde–Gly–squaramide assembly. Without **1**, the aldehydes, although significantly more active than the control compounds, are not particularly active. In the presence of **1** loaded at 1 mol % (transporter to lipid), **2** and **3** become reasonably active as mobile transporters, with EC_{50} values at low percentage loadings. It is interesting to note that although without **1**, the activity follows the sequence **10** > **2** ≈ **3**, the presence of **1** changed the sequence to **2** > **3** ≫ **10**. Possibly, fluorous interactions[35] between trifluomethyl groups of **1** and those of the aldehydes promoted the formation of the ternary Gly–squaramide–aldehyde conjugate, and it is reasonable that **2** interacts with **1** most efficiently as a result of similar positions of trifluoromethyl substituents. The significantly decreased activity with **11** and little activity with **12** are likely due to the lower electrophilicity (therefore slower rate for hemiaminal/imine formation) as well as the lower lipophilicity.

Transport of Other Substrates. The transport of other substrates, including sarcosine (Sar), *N*,*N*-dimethylglycine (DMG), oxalic acid/oxalate, and calcein, was studied in an attempt to reveal the transport mechanism in the case of Gly transport. Sar can form hemiaminals but not imines with aldehydes. Interestingly, a mixture of **1** (1 mol %) and **2** or **3** (10 mol %) promotes Sar transport synergistically [Figures 6a and S26 (Supporting Information)], similarly to the results found with Gly transport. This suggests that formation of the hemiaminal is sufficient for facilitated transport of amino-containing substrates, without the need for the dehydration step to form the imine, although it is still possible that transport by an imine intermediate is the dominant pathway in the case of Gly transport. It is also of interest to test the transport of DMG, which might form zwitterionic tetrahedral adducts[36] with aldehydes. The results (Figures 6b) show that DMG crosses the membrane by simple diffusion very quickly due to its lipophilicity. DMG diffusion is slightly enhanced by **1** (1 mol %), whereas the aldehyde component does not affect the transport [Figures 6b and S27 (Supporting Information)], which might be due to fast DMG simple diffusion making aldehyde-facilitated transport difficult to observe or the inability of the aldehydes to promote the DMG transport due to the instability of the zwitterionic tetrahedral adduct.

To further confirm that the aldehyde-facilitated Gly/Sar transport is due to the aldehyde interaction with the amino group, instead of with the carboxylate/carboxylic acid group or with the squaramide **1**, oxalic acid/oxalate transport was studied using a HPTS base-pulse assay.[37] POPC LUVs (mean diameter 200 nm) were loaded with the pH sensitive dye 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS, 1 mM) and

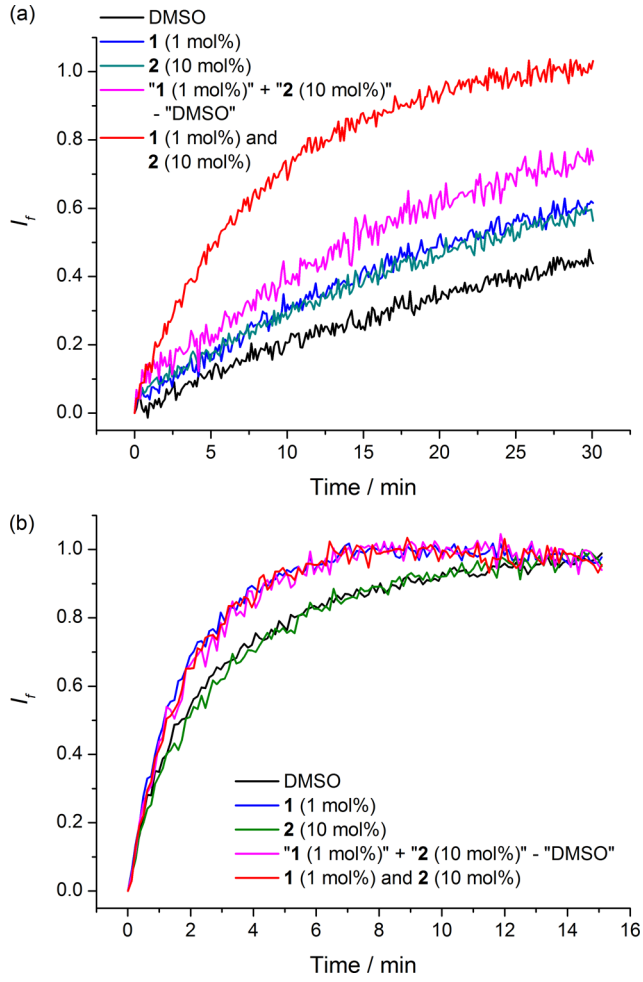

Figure 6. Transport kinetics of Sar (a) and DMG (b) measured by the Cu^{2+}–calcein assay. POPC LUVs (mean diameter 200 nm) loaded with $CuSO_4$ (0.2 mM) and calcein (0.2 mM) were suspended in an external solution containing Sar or DMG (30 mM) and $CuSO_4$ (0.2 mM). Both the internal and external solutions contained Na_2SO_4 (100 mM) and HEPES (20 mM) buffered at pH 7.4. At time 0, DMSO solutions of transporters or DMSO was added, and the fluorescence intensity (λ_{ex} = 495 nm, λ_{em} = 515 nm) was recorded. The fluorescence intensity was normalized by saturation using a mixture of **1** (1 mol %) and **3** (50 mol %) (a) or by using the maximum fluorescence intensity over the course of the measurement (b). Transporter loadings are shown as transporter to lipid molar ratios. Note that in part b there is a slight decrease of the fluorescence intensity after 10 min, due to the influx of Cu^{2+} ion when the DMG influx is close to completion. For a detailed discussion, see section 6 in Supporting Information.

sodium oxalate (100 mM), suspended in an external solution containing sodium oxalate (100 mM). A pulse of NaOH (5 mM) was applied to generate a transmembrane pH gradient, and the dissipation of the pH gradient in the absence and presence of transporters was monitored. The dissipation of the pH gradient can be due to the transport of neutral oxalic acid, oxalate (or hydrogenoxalate) transport coupled to OH^- (or H^+) transport, Na^+ transport coupled to H^+ (or OH^-) transport, HEPES transport, HPTS transport, or the detergent effect. As shown in Figures 7 and S30 (Supporting Information), **1** (1 mol %) induces slow transport, while **2** or **3** (10 mol %) induces no transport and has little influence on the transport induced by **1**. The HPTS response was found to

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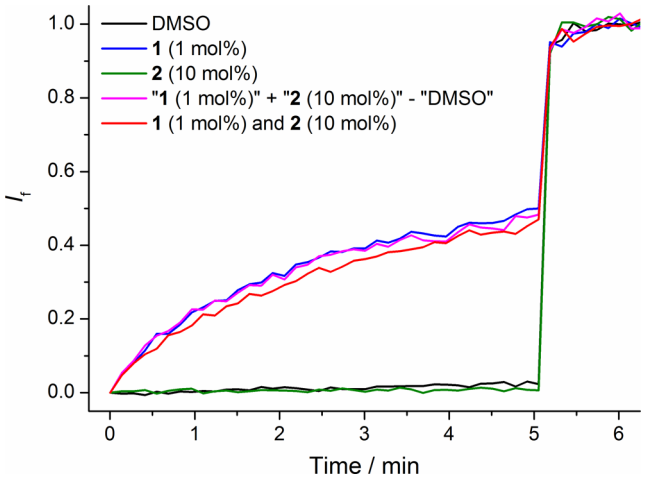

Figure 7. Oxalic acid/oxalate transport kinetics studied by a HPTS assay. POPC LUVs (mean diameter 200 nm) were loaded with HPTS (1 mM). Both the internal and external solutions contain sodium oxalate (100 mM) and HEPES (10 mM) buffered at pH 7.0. DMSO solutions of transporters or DMSO was added, followed by a pulse of NaOH (5 mM) at time 0, and the fluorescence ratio of HPTS (λ_{ex} = 460 nm, λ_{em} = 510 nm, base form vs λ_{ex} = 403 nm, λ_{em} = 510 nm, acid form) was recorded. The fluorescence ratio was normalized by calibration using a detergent to lyse the vesicles at 5 min.

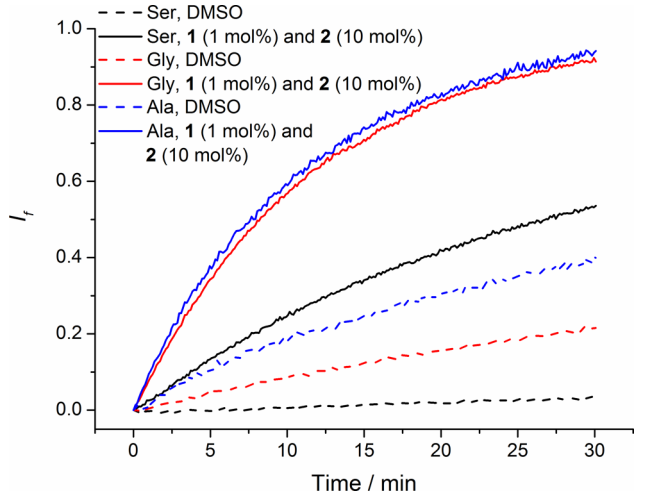

Figure 8. Transport kinetics of Ser, Gly, and Ala measured by the Cu^{2+}–calcein assay. POPC LUVs (mean diameter 200 nm) loaded with $CuSO_4$ (0.2 mM) and calcein (0.2 mM) were suspended in an external solution containing the amino acid (30 mM) and $CuSO_4$ (0.2 mM). Both the internal and external solutions contained Na_2SO_4 (100 mM) and HEPES (20 mM) buffered at pH 7.4. At time 0, DMSO solutions of transporters or DMSO was added, and the fluorescence intensity (λ_{ex} = 495 nm, λ_{em} = 515 nm) was recorded. The fluorescence intensity was normalized by saturation using a mixture of **1** (1 mol %) and **3** (50 mol %). Transporter loadings are shown as transporter to lipid molar ratios.

be minor when the anion in both the internal and external solutions was changed from oxalate to the more hydrophilic SO_4^{2-} (Figure S31, Supporting Information), which proves that the HPTS response observed with the sodium oxalate system results, at least predominantly, from the transport of oxalic acid, hydrogenoxalate, or oxalate. It is thus unlikely that the role of the aldehyde component in facilitating the transport of Gly/Sar is the interaction with the carboxylate/carboxylic group or "activating" the squaramide **1**.

A calcein leakage assay[38] was also performed using POPC LUVs entrapping self-quenched calcein dyes (100 mM) to test the integrity of the vesicle membrane. No leakage of calcein dye was detected (Figure S34, Supporting Information), leading us to suggest that Gly transport occurs with no disruption of the vesicle structure and no formation of large transmembrane pores.

A mixture of **1** (1 mol %) and **2** (10 mol %) was also tested to facilitate the transport of alanine (Ala) and serine (Ser). The synergistic effect between **1** and **2** was also observed (Figures S28 and S29, Supporting Information). The rates of transport of the three tested amino acids with primary amino groups facilitated by the **1**–**2** mixture follow the order Ala ≈ Gly > Ser (Figure 8), which is consistent with the higher hydrophilicity of Ser.

^{1}H NMR Studies. The ability of the aldehyde component to form hemiaminals or imines was examined using a biphasic experiment. A $CDCl_3$ solution of **2** (10 mM) was mixed with an aqueous solution of Gly (or Sar, 1 M) and tetrabutylammonium (TBA) salt of Gly (or Sar) anion (1 M). After vortexing of the mixture for 5 min, the $CDCl_3$ phase was subject to ^{1}H NMR measurements. The use of a Gly–TBAGly or Sac–TBASar mixture instead of TBAGly or TBASar alone is to prevent the aqueous solution from being too basic, thus reducing undesired side reactions. With the Gly–TBAGly system (Figure 9, upper panel), both hemiaminals and imines can be observed in the ^{1}H NMR spectra of the $CDCl_3$ phase, along with the free aldehyde, the hydrate, and the carboxylate that results from aldehyde oxidation under basic conditions. The Gly hemiaminal shows the CH proton resonance at δ = 5.45 ppm and two doublets for the methylene proton resonances between δ = 3.45 and 3.65 ppm, which indicates the creation of a tetrahedral stereocenter. The Gly imine shows its CH proton resonance at δ = 8.35 ppm, which overlaps with the peak from one set of aldehyde aromatic protons, and a singlet peak for the methylene protons at δ = 4.37 ppm. With the Sar–TBASar system (Figure 9, lower panel), the hemiaminal is formed and shows also two doublets for the diastereotopic methylene protons. It is interesting to note that hemiaminals are normally not observed due to the instability. The presence of the hemiaminals observed in our systems results from the high electrophilicity of the aldehyde carbonyl group due to the presence of two electron-withdrawing trifluoromethyl groups. The coexistence of the hemiaminal and the imine in the case of the Gly–TBAGly–**1** system might suggest that Gly transport facilitated by aldehydes could occur by a mixture of hemiaminal and imine intermediates, although currently there has been no direct evidence for that hypothesis. When the aqueous phase contains only Gly or Sar (2 M), no hemiaminal or imine is detectable in the $CDCl_3$ phase (Figure S35, Supporting Information), which is consistent with the reported low equilibrium constants for the formation of protonated hemiaminals[16d] (**H2** and **H3** in the case of Gly) and protonated imines[39] (**I2** and **I3**) without stabilization by an intramolecular hydrogen bond.[40]

Transport Mechanism. The Gly transport mechanism was examined using vesicles of different lipid compositions. The transport activity was found to decrease very slightly with POPC–cholesterol (7:3, molar ratio) LUVs (mean diameter 200 nm) compared to that with POPC LUVs (mean diameter 200 nm) (Figure S22, Supporting Information). Cholesterol is known to decrease the fluidity of the lipid membrane in the

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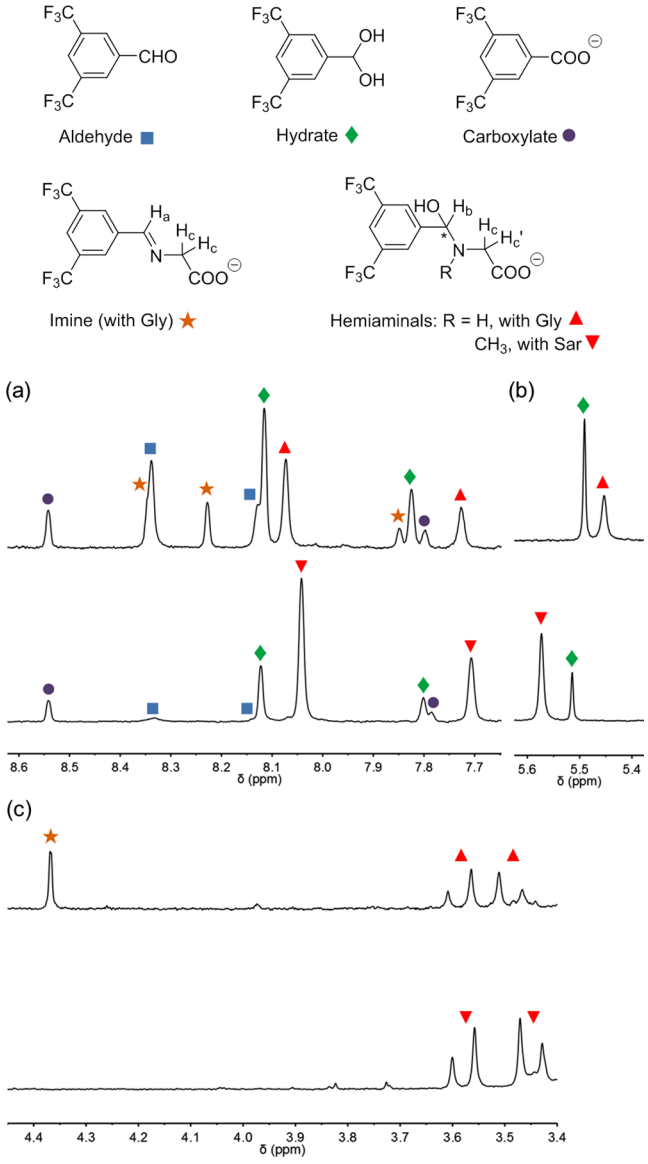
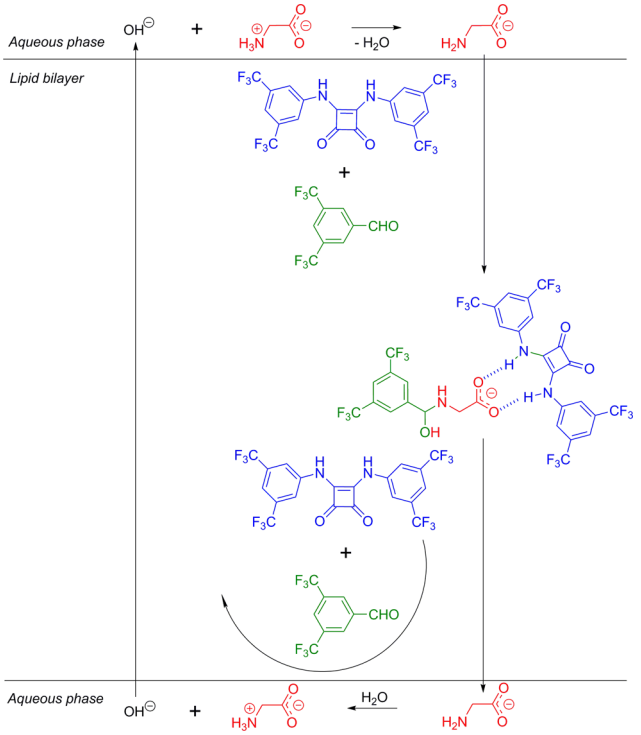


Figure 9. Partial ^{1}H NMR spectra (400 MHz) of $CDCl_3$ solutions of **1** (10 mM) after treatment with an aqueous solution containing a mixture of Gly (1 M) and TBAGly (1 M) (upper panel), or Sar (1 M) and TBASar (1 M) (lower panel): (a) downfield region showing aromatic protons and the imine CH proton H_a, (b) midfield region showing CH protons (H_b) of the hemiaminals and the hydrate, and (c) upfield region showing methylene protons (H_c) of the hemiaminals and the Gly imine.

liquid phase, thus decreasing the activity of a mobile transporter.[41] However, enhanced partition of a lipophilic transporter by cholesterol[6c] could also occur. The observed minor influence of cholesterol is possibly a result of both effects. With dipalmitoylphosphatidylcholine (DPPC) LUVs (mean diameter 200 nm) (Figure S23, Supporting Information), Gly transport was suppressed at 37 °C, which is below the main phase transition temperature of 41 °C, while it is switched on at 45 °C, which might support a mobile carrier mechanism. The transport activity of the squaramide–aldehyde mixtures decreased markedly in LUVs (mean diameter 200 nm) of an anionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) as compared to POPC LUVs (mean diameter 200 nm) (Figures S24a and S24b, Supporting Information), which is consistent with the transport of an anionic species that experiences electrostatic repulsion with the anionic lipid headgroup,[7b] although binding of **1** to the anionic headgroup of POPG could also account for the decreased activity. These results, together with the Hill coefficients (Table 1), the absence of nonspecific leakage (Figure S34, Supporting Information), and also the small size of the tested aldehydes, support a mobile carrier mechanism for the observed facilitated Gly transport. Transport by a zwitterionic hemiaminal (**H2**) or imine (**I2**) is not favorable, because of the relative instability of the adducts when the hemiaminal nitrogen[16d] or imine nitrogen[39,40] is protonated as compared to the deprotonated species (**H1** and **I1**) and also the hydrophilicity of the zwitterionic species. Therefore, the transport facilitated by the squaramide–aldehyde mixtures is proposed to occur via the anionic aldehyde–glycine hemiaminal (**H1**) or imine (**I1**) with the negatively charged carboxylate group hydrogen bonded to **1** (Scheme 2 shows the hemiaminal pathway). Interestingly, the

Scheme 2. Proposed Mechanism for Gly Transport Facilitated by 1 and 2 Forming a Three-Component Assembly Involving Hemiaminal Formation[a]



[a]Alternatively, imine formation is possible. OH^- transport is facilitated by **1**, which is not shown.

loss of activity in POPG vesicles is not observed with a high concentration of aldehyde transporters without **1** (Figures S24c and S24d, Supporting Information), suggesting that in this case, the transported species is most likely the neutral aldehyde–glycine adduct (whether hemiaminal **H3** or imine **I3**), although it is less stable than the anionic adducts, which might partly account for the lower transport activity of aldehydes when **1** is absent. Similarly, the anionic vesicles did not decrease the rate of Gly simple diffusion (Figure S24, Supporting Information), consistent with reported assignment of the simple diffusion to movement of the zwitterionic species through bilayer fluctuations and transient defects or partitioning of the neutral

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H_2NCH_2COOH, which is in equilibrium with the zwitterionic species.[42]

The movement of the anionic form of Gly facilitated by a squaramide−aldehyde mixture would need to be accompanied by cotransport with a cation or antiport with an anion. The dominant cation and anion present in our system are Na^+ and SO_4^{2-}, respectively. Because of the basicity of the Gly anion (pK_a of Gly ammonium group is 9.60), cotransport with Na^+ or antiport with SO_4^{2-} or HSO_4^- would lead to intravesicular basification. However, a HPTS assay (Figure S32, Supporting Information) shows that the internal pH change induced by 30 mM Gly is negligible (<0.05 pH unit, with 5 mM HEPES buffer at pH 7.4), indicating H^+/H_2NCOO^- symport or functionally equivalent H_2NCOO^-/OH^- antiport, the net result being transport of the overall neutral Gly (Scheme 1). It seems more plausible to describe the transport as a H_2NCOO^-/OH^- antiport, given the potential of **1** to bind OH^- by hydrogen bonding. It is still possible that a metal ion is cotransported with the Gly anion and the resulting pH gradient is being compensated by M^+/H^+ antiport or M^+/OH^- symport in the same direction or X^-/OH^- antiport or X^-/H^+ symport in the opposite direction. Changing the external metal ion from Na^+ to the more easily dehydrated Cs^+ has little influence on the Gly transport activity (Figure S25, Supporting Information), suggesting the minor, if any, role of metal ion transport.

CONCLUSIONS

In summary, we have combined a dynamic covalent bond and a noncovalent interaction in the design of a transmembrane transport system for amino acids. A dynamic three-component assembly involving a squaramide hydrogen bonding to the Gly carboxylate group and an aldehyde forming a hemiaminal or a imine linkage with the Gly amino group is assumed to be responsible for reversibly binding and releasing Gly in the lipid bilayers. The transport process is proposed as H_2NCOO^-/OH^- antiport. Several negative control experiments and a biphasic ^1H NMR study support the role of aldehyde carbonyl group addition in the facilitated transport. Although it is currently not confirmed whether Gly transport facilitated by aldehydes proceeds via a hemiaminal or an imine intermediate, or via both pathways, the ability of hemiaminal formation to facilitate membrane transport has been revealed by the observation of Sar transport facilitated by a squaramide−aldehyde mixture working in a synergistic manner. Thus, hemiaminal formation has been demonstrated as a new interaction for facilitated membrane transport. This might have implications for transmembrane delivery of larger cargos that contain primary or secondary amine groups. A rapid and easy fluorescence assay for amino acid transport has been developed that allows monitoring of fast transport kinetics. This assay is suitable for amino acid transporters that do not transport Cu^{2+}, which can be easily identified.

ASSOCIATED CONTENT

Supporting Information

Vesicle transport assay details, kinetic data, Hill plots, and other supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*philip.gale@soton.ac.uk

Notes

The authors declare no competing financial interest.

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